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Initial Steps in the Degradation of Phosphinothricin (Glufosinate) by Soil Bacteria

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Three hundred bacterial isolates from soil were tested for resistance against phosphinothricin [PPT; DL-homoalanin-4-yl(methyl)phosphinic acid], the active ingredient of the herbicide BASTA. Eight resistant bacterial strains and *Escherichia coli* were analyzed for PPT-transforming activities. At least three different enzymatic reactions could be detected in cell extracts. In six strains an acetyltransferase was active, synthesizing *N*-acetyl-PPT in the presence of PPT and acetyl coenzyme A. All strains could degrade PPT to its corresponding 2-oxoacid {2-oxo-4-[(hydroxy)(methyl)phosphinoyl] butyric acid} by transamination. *Rhodococcus* sp., the only tested strain that was able to utilize PPT as a sole source of nitrogen, formed 2-oxo-4[(hydroxy)(methyl)phosphinoyl]butyric acid by oxidative deamination. This enzymatic activity was inducible by L-glutamic acid or PPT itself but not in the presence of NH_4^+. D-PPT transformation was not detectable in any of the investigated strains.

Phosphinothricin [PPT; DL-homoalanin-4-yl(methyl)phosphinic acid] is produced as part of a dialanyl tripeptide by several *Streptomyces* strains. It was first isolated from *Streptomyces viridochromogenes* and characterized as an antibiotic (1). The compound also has herbicidal activity and is the active ingredient of the herbicide Hoe 39866 (HOECHST AG) with the proposed common name glufosinate ammonium. PPT was shown to be a potent inhibitor of glutamine synthetase in *Escherichia coli* (1, 3) and plants (7, 13). Its herbicidal action is due to the light-dependent accumulation of ammonia (11, 24). Soil studies suggest that PPT is rapidly inactivated and biologically transformed to degradation products (17). As the major catabolite in soil 3-methylphosphinico-propanoic acid was identified (8).

So far nothing is known about the effect of PPT on growth of soil microorganisms and the strains and enzymes involved in its metabolism. Therefore an investigation was conducted to determine the degree of PPT resistance and utilization among soil bacteria and to study its degradation in isolated strains from soil under laboratory conditions. In this article we describe the formation of two PPT metabolites by three different enzymatic reactions in nine strains investigated. The initial steps of PPT degradation by soil bacteria and their role in PPT resistance and utilization are discussed.

MATERIALS AND METHODS

Isolation and selection of soil microorganisms. Soil samples (clay loam soil) were taken from a barley field (Hattersheim, Federal Republic of Germany) that had been exposed to two successive annual applications of BASTA (commercial formulation of the herbicide PPT) (HOECHST AG). One gram of soil was extracted with 10 ml of 0.5% sodium hexametaphosphate solution for 1 h at room temperature. The solution was plated in a series of dilutions on agar plates containing 0.5% yeast extract and 2 mM glucose. Single colonies appeared after 2 to 3 days of incubation at 28°C. A total of 300 colonies were isolated and screened for resistance against PPT on agar plates as well as in liquid cultures by growing them for 5 days at 28°C in a medium containing 5 mM glucose, 5 mM succinate, 10 mM glycerol, and Winogradski mineral salts (16). The pH was adjusted to 7.2. L-PPT was added at concentrations of 0.1, 1, 10, 20, and 40 mM.

Microbial characterization. The bacterial strains chosen for the PPT degradation experiments and enzyme studies were characterized by Gram staining and light microscopy and identified by using the semiautomated microbial identification test strips API 20E (for *Enterobacteriaceae*) and API 20NE (for non-*Enterobacteriaceae*) from API bio Merieux, Nürtingen, Federal Republic of Germany. Isolation and identification of *Rhodococcus* sp. strain DX-35 and *Pseudomonas paucimobilis* FX-90 have been described earlier (19).

Degradation experiments with resting cells. Bacterial cells were harvested in the mid-exponential growth phase by centrifugation (8,000 × *g*, 12 min, 4°C), washed with 50 mM potassium phosphate buffer (pH 7.2)–80 mg of chloramphenicol per liter, and suspended in the same buffer. The test solutions contained cell suspension (0.2 to 0.5 mg of protein per ml), 1 mM 3,4-^{14}C-labeled DL-PPT, and, in the inhibition experiments, 10 mM aminooxyacetic acid (AOA). The volume was 1 to 5 ml, depending on the number of samples taken. The incubation was performed in a shaking water bath (30°C, 2 Hz). Samples of 500 μl were withdrawn at 20-min intervals, and cells were removed by centrifugation. After a passage through membrane filters (pore size, 0.2 μm) the samples were analyzed by high-pressure liquid chromatography on ion-exchange columns (250 by 4.6 mm; Nucleosil SB; Macherey and Nagel, Düren, Federal Republic of Germany) with Na_2SO_4 as an eluent. PPT and degradation products were detected and quantified by a radioactivity monitor with a glass solid scintillator (Ramona D; Isomess, Straubenhardt, Federal Republic of Germany).

Preparation of cell extracts. Soil isolates were grown in mineral salts medium (400-ml cultures) for 2 days under the conditions described above. Cells were harvested by centrifugation, washed twice in 10 mM NaCl–10 mM NaH_2PO_4 (pH 7.0), and suspended at 1.7 ml/g of cells in the same buffer. Cell suspensions were disrupted by sonication for 20 5-s periods with a Branson sonifier (model B 15). The sonication vial was kept on ice during the whole procedure. Intact

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bacteria and cell debris were removed by centrifugation at 8,000 × *g* for 20 min at 4°C. Supernatants were dialyzed overnight at 4°C against 50 mM NaH_2PO_4 buffer (pH 7.5). The protein concentration was determined by using the Coomassie brilliant blue binding assay (Bio-Rad Laboratories, Richmond, Calif.) (2). Cell extracts could be stored for several weeks at −20°C without loss of enzymatic activities.

Degradation experiments with cell extracts. PPT degradation experiments were performed by incubating 50 μl of cell extracts either with 3,4-^{14}C-labeled DL-PPT in the presence of various cosubstrates or with nonradioactive PPT and 1-^{14}C-labeled acetyl coenzyme A (acetyl-CoA). Assay conditions and substrate concentrations are given in the legends of Fig. 1 and 2. Reactions were stopped by the addition of 1 volume of 12% trichloroacetic acid, kept on ice for 30 min, and centrifuged at 8,000 × *g* for 20 min to remove denatured proteins. Samples (7 μl) of the supernatant fractions were separated by thin-layer chromatography on HPTLC-cellulose plates (E. Merck AG, Darmstadt, Federal Republic of Germany) with *n*-propanol–25% NH_3 (3:2, vol/vol) as the solvent. PPT degradation products were visualized by autoradiography and identified by cochromatography with standard substances.

Identification of PPT metabolites. The identity of PPT metabolites was confirmed by high-pressure liquid chromatographic analysis. Degradation assays were carried out as described above but using only unlabeled substrates. After incubation, 0.5 volume of 6 N H_2SO_4 was added, and denatured proteins were removed by centrifugation. The organic acids in the supernatant were separated on a Bio-Rad Aminex ion-exclusion column HPX 87 H in 0.01 N H_2SO_4 with a flow rate of 0.5 ml/min and detected with a variable-wavelength monitor (LKB Instruments, Inc., Rockville, Md.) at 190 nm. PPT metabolites were identified by comparison with standard substances. The retention times were 9.3 min for 2-oxo-4-[(hydroxy)(methyl)phosphinoyl] butyric acid (PPO) and 14.1 min for *N*-acetyl-PPT.

Enzyme assay of L-PPT *N*-acetyltransferase. L-PPT *N*-acetyltransferase was assayed quantitatively by incubating cell extracts containing 100 mM Tris hydrochloride (pH 8.2) for 1 h with 10 mM L-PPT in the presence of 2 mM acetyl-CoA at 30°C. Samples of 25 μl were taken at 10-min intervals and analyzed for *N*-acetyl-PPT formation by high-pressure liquid chromatography as described above. Specific enzyme activities were calculated in milliunits per milligram of protein (1 U is equal to 1 μmol of *N*-acetyl-PPT formed per min).

Enzyme assay of L-PPT oxidase. L-PPT oxidase activity was determined by measuring the release of NH_4^+ from L-PPT. Cell extracts in 50 mM phosphate buffer (pH 7.5) were incubated for 1 h either with 1 mM DL-PPT or 9 mM L-PPT at 30°C. A control reaction was included containing no PPT. Samples of 200 μl were taken at 10-min intervals and analyzed spectrophotometrically for NH_4^+ content (23) (1 U is equal to 1 μmol of NH_4^+ released per min).

Oxygen consumption was determined by the conventional Warburg technique (21). Warburg flasks contained in the main compartment 1.5 ml of cell extract (1.7 mg of protein) and in the sidearm 20 μmol of DL-PPT in 200 μl of water. Adsorption solutions for CO_2 were omitted. After the reaction was started by the addition of PPT to the main compartment, O_2 consumption was followed for 60 min at 28°C. At the end of the experiment PPT-released NH_4^+ was determined spectrophotometrically.

Enzyme assay of L-PPT transaminase. L-PPT transamination was quantified by measuring the formation of L-PPT from PPO in the presence of a donor amino acid. Cell extracts in phosphate buffer (pH 7.5) were incubated for 1 h with 10 mM PPO, 2 mM pyridoxalphosphate (PLP), and 40 mM of a donor amino acid at 30°C. Samples of 25 μl, taken at 10-min intervals, were boiled for 10 min. After removal of denatured proteins by centrifugation, the supernatants were analyzed for L-PPT with Biotronic Amino Acid Analyzer LC 5001 by using a 3.2- by 130-mm BTC-2710 column (1 U is equal to 1 μmol of L-PPT formed per min).

TABLE 1. PPT-metabolizing activities of resting cells of *Rhodococcus* sp. cultured with different nitrogen sources and inhibition in the presence of 10 mM AOA

N source[a]	PPT-transforming activity (mU/mg of protein)	% Inhibition with AOA
L-PPT	21.2	38.7
L-Glu	21.7	26.4
NH_4^+	2.2	100.0

[a] Initial concentration in the culture medium was 2 mM.

Materials. The racemic mixture of PPT (DL-PPT), its NH_2 enantiomers (L-PPT and D-PPT), and the PPT derivates PPO and *N*-acetyl-PPT were obtained from HOECHST AG, Frankfurt/Main, Federal Republic of Germany. The ^{14}C-labeled compounds [3,4-^{14}C]PPT, [3,4-^{14}C]PPO, and [3,4-^{14}C]*N*-acetyl-PPT (specific activities: 1.8×10^8 to 3.7×10^8 Bq mmol^{-1}) were from HOECHST AG as well; [1-^{14}C]acetyl-CoA (2.0×10^9 Bq mmol^{-1}) was purchased from Amersham Buchler, Braunschweig.

RESULTS

PPT-susceptible and PPT-resistant bacteria. To determine the degree of PPT resistance among soil microorganisms, bacterial isolates from a PPT-treated soil were cultivated on agar plates containing a complete mineral medium with L-PPT added at different concentrations (0.1 to 40 mM). More than 90% of 300 isolates tolerated concentrations as high as 40 mM, whereas less than 5% were inhibited by PPT concentrations above 1 mM. The most susceptible bacterium, however, was the laboratory strain *E. coli* DH-1, which therefore was included in our investigations. Resistant microorganisms could be differentiated into two groups: one group was able to utilize L-PPT as a sole source of nitrogen, whereas the other was not. As members of the first group *Rhodococcus* sp. and *Pseudomonas paucimobilis* were identified in an earlier investigation (19). *Agrobacterium tumefaciens*, *Alcaligenes* sp., *Alcaligenes faecalis*, *Pseudomonas* sp., *Serratia plymuthica*, *Enterobacter* sp., and *Enterobacter agglomerans* tolerated high concentrations of PPT but could not utilize the compound as a nitrogen source.

Transformation of PPT by resting cells. The metabolism of PPT was first studied in resting cells of *Rhodococcus* sp., a strain representing the group of L-PPT-utilizing bacteria, which therefore was expected to express PPT-transforming activity. Intact cells of *Rhodococcus* sp. were able to transform PPT into PPO, the corresponding 2-oxoacid of the compound. However, deamination rates did not exceed 50% of the applied racemic mixture of DL-PPT, indicating a specificity of the PPT-transforming reaction only for the L isomer of the substrate. The specific PPT-transforming activity in cells of *Rhodococcus* sp. depended on the nitrogen source in the culture medium (Table 1). In cells grown with L-glutamate, PPT transformation rates were similar to those with L-PPT itself, but with NH_4^+ they decreased drastically.

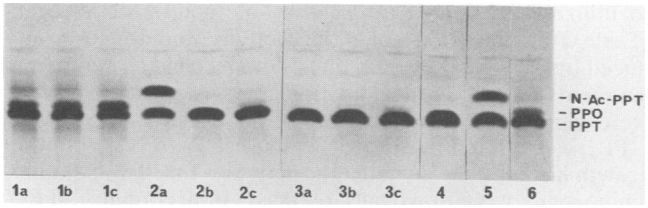

FIG. 1. TLC analysis of 3,4-^{14}C-labeled DL-PPT and metabolites obtained by dialyzed cell extracts of three bacterial strains, all cultivated in a mineral medium: *Rhodococcus* sp. (lanes 1) with 10 mM L-PPT as a sole source of nitrogen, *A. faecalis* (lanes 2) with additional 10 mM L-PPT, and *E. coli* (lanes 3) without PPT. Three different assays were performed with every extract: a, with 5 mM acetyl-CoA; b, without any cofactor; c, with 5 mM 2-oxoglutaric acid and 2 mM PLP. Each test contained 1.25 mM PPT as a substrate; protein concentrations were 1 mg ml^{-1}. Analysis was performed after 1 h of incubation at 30°C. Lanes 4 through 6 show the ^{14}C-labeled references PPT (R_f, 0.18), PPT with *N*-acetyl-PPT (R_f, 0.23), and PPT with PPO (R_f, 0.20), respectively). The origin is at the bottom of the photograph.

Moreover, the addition of AOA, an inhibitor of pyridoxalphosphate (PLP)-dependent enzymes (9), resulted in a reduction of PPT transformation. In cells precultured on L-PPT or L-glutamate, only a partial inhibition was determined, whereas in cells grown with NH_4^+ the PPT-transforming activity was blocked completely by AOA.

From these results one could conclude that at least two different enzymes participated in the oxidative deamination of PPT in *Rhodococcus* sp., one being PLP dependent and the other being regulated by the nitrogen source in the culture medium.

Formation of PPT metabolites by cell extracts. For further investigation of the PPT-transforming reactions in *Rhodococcus* sp., the following experiments were carried out with cell extracts instead of whole cells, thus avoiding the cell wall barrier between enzymes and substrates and allowing the performance of enzyme assays under defined reaction conditions and in the presence of various cosubstrates. To find out whether the resistance against PPT was mediated by the ability to transform the compound into nontoxic metabolites, seven bacterial isolates from soil that were unable to utilize L-PPT as a nitrogen source but characterized by a very high level of PPT resistance were included in the experiments. In addition *E. coli* DH1 was chosen as an example of a PPT-susceptible strain.

Thin-layer chromatography (TLC) analyses of metabolites for three typical strains are given in Fig. 1. PPO, the corresponding 2-oxoacid of PPT, was identified as the main catabolite of *Rhodococcus* sp. Its formation did not depend on the addition of any cofactor. Small amounts of *N*-acetyl-PPT could also be detected (Fig. 1, lanes 1a through c). In *A. faecalis*, a PPT-resistant strain, *N*-acetyl-PPT formation was the major PPT-transforming reaction. It depended on the addition of acetyl-CoA. Without any cofactor no transformation was detectable, but with 2-oxoglutaric acid and PLP small amounts of PPO were formed (Fig. 1, lanes 2a through c). Even in cell extracts of *E. coli*, a PPT-susceptible bacterium, PPO was produced when 2-oxoglutaric acid and PLP had been added (Fig. 1, lanes 3a through c). In all strains tested, at least one-half of the radioactive DL-PPT added to the assay mixtures remained unchanged, suggesting that the PPT-transforming activities were specific for only one isomer of the racemate.

Properties of PPT-metabolizing enzymes. (i) PPT *N*-acetyltransferase. To confirm the identity of *N*-acetyl-PPT as a metabolite of PPT, cell extracts of *A. faecalis* were incubated with L- or D-PPT and [1-^{14}C]acetyl-CoA as the acetyl donor. TLC analyses revealed the formation of an acetylation product with L-PPT. This product was identified as *N*-acetyl-PPT by cochromatography with a standard substance. No product was observed when L-PPT was replaced by D-PPT, indicating that the reaction was specific for the L isomer (Fig. 2). Only acetyl-CoA could serve as an acetyl donor. With other acetyl donors, e.g., acetylphosphate, acetylornithine, or acetylglutamate, no PPT transformation was detectable (data not shown).

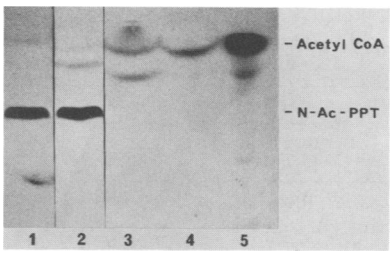

FIG. 2. TLC analysis of PPT *N*-acetylation assay with cell extracts of *A. faecalis* in the presence of 0.15 mM 1-^{14}C-labeled acetyl-CoA. Lanes: 1, *N*-acetyl-PPT as a standard; 2, with 2 mM L-PPT; 3, with 2 mM D-PPT; 4, without PPT; 5, acetyl-CoA (R_f, 0.41) as a standard. For assay conditions see the legend to Fig. 1.

The ability of *Rhodococcus* sp. to synthesize *N*-acetyl-PPT did not depend on the presence of PPT in the culture medium. Grown with NH_4^+ as a nitrogen source, the strain produced *N*-acetyl-PPT (Fig. 3, lane 1). However, the rate of *N*-acetyl-PPT formation of *A. faecalis* cell extracts could be increased about 10-fold to a specific activity of 20 mU/mg of protein when the culture medium contained additional 10 mM L-PPT. Similar results were obtained for *Alcaligenes denitrificans*. Six out of nine strains investigated were capable of synthesizing *N*-acetyl-PPT when L-PPT and acetyl-CoA were added to the cell extracts.

(ii) PPT oxidase. In the absence of any cofactor, cell extracts of *Rhodococcus* sp. showed a PPT-deaminating activity when the strain was grown on PPT as a nitrogen source. The rate of NH_4^+ release could not be increased by the addition of NAD^+ or $NADP^+$. In a Warburg experiment, 0.7 mmol of O_2 was consumed per mmol of PPT, suggesting that the deamination of PPT was performed by an oxidase rather than by a dehydrogenase. Theoretically a ratio of 0.5 would be expected. When cultivated on PPT as a nitrogen source, cell extracts of *Rhodococcus* sp. incubated with 9 mM L-PPT were able to release NH_4^+ at a specific activity of 80 to 120 mU/mg of protein. In enzyme assays performed

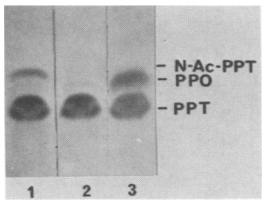

FIG. 3. TLC analysis of 3,4-^{14}C-labeled DL-PPT and metabolites obtained by cell extracts of *Rhodococcus* sp., cultured on NH_4^+ as a nitrogen source. For assay conditions see the legend to Fig. 1. With acetyl-CoA (lane 1), the synthesis of *N*-acetyl-PPT could be detected; without any cofactors (lane 2), no product was observed, but when 2-oxoglutarate and PLP were added (lane 3), formation of PPO was seen.

TABLE 2. Relative NH_4^+ release activities of amino acids with cell extracts of *Rhodococcus* sp. cultivated with different nitrogen sources

Test substrate[a]	% Activity with the following nitrogen source:		
	DL-PPT (5 mM)	L-Glu (2.5 mM)	NH_4^+ (2.5 mM)
L-PPT	100.0[b]	99.7	9.8
D-PPT	6.3	8.1	5.6
L-Glu	98.2	89.4	11.4
L-Gln	212.1	254.9	15.4
L-Val	40.2	36.0	15.4
L-Ile	45.8	41.3	9.8
L-Ser	90.6	94.0	1.7
L-Ala	180.7	172.1	4.1
L-Phe	107.4	129.5	2.5
L-His	148.9	163.6	1.7
L-Asp	32.7	32.2	2.8
L-Asn	78.0	81.7	18.7
L-Arg	263.0	278.0	1.3
L-Met	148.8	189.2	16.7
L-Cys	104.3	109.2	13.2
L-Tyr	31.9	69.4	3.8
L-Lys	291.9	313.2	39.4
L-Trp	104.1	113.0	7.4
L-Leu	265.6	292.6	32.2
L-Thr	0.0	4.8	0.0
L-Pro	2.8	1.0	0.7
Gly	4.1	5.8	0.0

[a] Concentration of test substrates was 9 mM; protein concentrations ranged from 0.8 to 1.0 mg ml^{-1}.

[b] L-PPT deamination of cells cultivated with DL-PPT is referred to as 100%.

with 1 mM DL-PPT the oxidase activity dropped to 40 mU/mg of protein. This was still two times the total PPT-transforming activity measured with resting cells under the same conditions (Table 1). The lower activity in intact cells compared with cell extracts might be due to a reduced contact between substrate and enzyme caused by the bacterial cell wall.

Besides PPT, 17 other amino acids could also be deaminated, 7 of them at rates even higher than that with PPT as a substrate. The low rates of NH_4^+ release measured with the test substrates D-PPT, L-threonine, L-proline, and glycine were expected because of the inability of the organism to utilize these amino acids as a sole source of nitrogen (Table 2). The PPT-deaminating activity could also be induced by growing the strain on L-glutamic acid as a nitrogen source. The specific deamination rates of all amino acids were similar, irrespective of whether L-glutamic acid or L-PPT was offered as a nitrogen source. With NH_4^+ in the growth medium, the deaminating activities for PPT and other amino acids were drastically reduced. In accordance, TLC analyses showed that NH_4^+-grown cells could not oxidize PPT to PPO without any cofactor (Fig. 3, lane 2). Diauxic utilization of nitrogen sources was observed in medium containing both NH_4^+ and PPT; the NH_4^+ was used first. PPT oxidase activity was detectable only when the NH_4^+ concentration was below 0.02 mM (data not shown).

(iii) PPT transaminase. Cell extracts of all investigated strains were able to degrade PPT to PPO when 2-oxoglutaric acid and PLP were added (Fig. 1 and 3), indicating the presence of a PPT-transforming aminotransferase. Since transaminase-catalyzed reactions are known to be reversible, the ability of all strains to synthesize PPT from PPO and L-glutamic acid in the presence of PLP confirmed the existence of a PPT-transaminating activity. The conversion of PPO to PPT was completely blocked either by the addition of 1 mM AOA or in the absence of an NH_2 donor. Accordingly, in resting cells of *Rhodococcus* sp. grown with NH_4^+ as a nitrogen source, which lack the oxidase activity, the remaining PPT-transforming reaction could be inhibited up to 100% by the addition of 10 mM AOA (Table 1). Inhibition of transamination in living cells by AOA has also been reported for phenylglycine transaminase in *Pseudomonas putida* (22).

Glutamic acid and glutamine were the only protein amino acids serving as effective amino group donors in *Rhodococcus* sp. and *E. coli*, whereas in *A. faecalis* some other amino acids also showed detectable activities. However, the highest activities for all three strains tested were observed with L-glutamic acid (Fig. 4). D-Amino acids were inactive in the transamination reaction.

The stereospecifity of PPT synthesis by enzymatic transamination could be demonstrated by incubating PPT, formed through transamination, with cell extracts from *A. faecalis* and an excess of acetyl-CoA. The PPT was quantitatively acetylated, indicating that the transaminase, like the other two PPT-metabolizing enzymes, was specific only for the L isomer of the compound (data not shown).

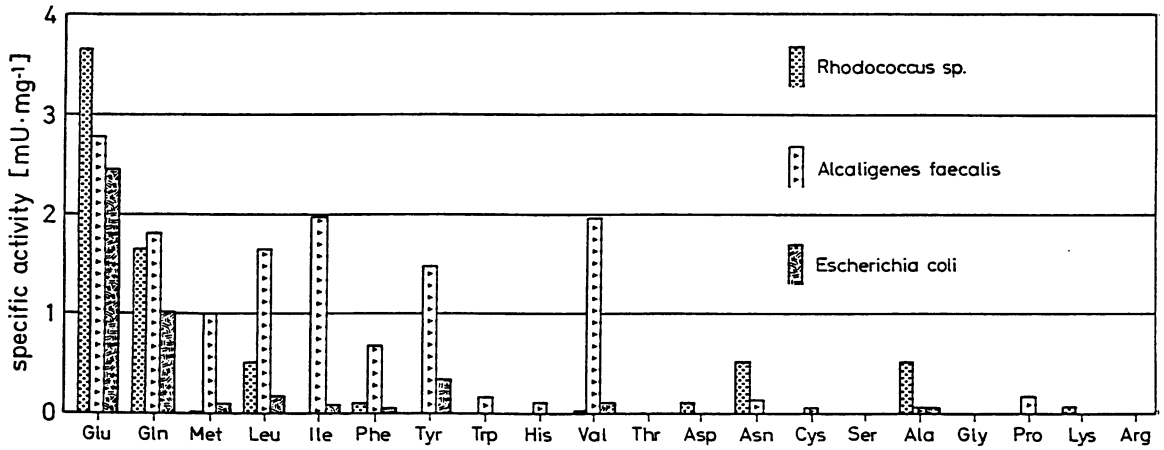

FIG. 4. PPT synthesis by transamination with PPO and different donor amino acids in cell extracts of *Rhodococcus* sp., *A. faecalis*, and *E. coli*. For cultivation conditions see the legend to Fig. 1; for assay conditions see Materials and Methods.

TABLE 3. PPT-transforming activities in investigated bacterial strains

Phenotypic character	Strain	PPT-transforming activity		
		Oxidase[a]	*N*-Acetyltransferase	Transaminase
PPT resistant, growth on PPT as N source	*Rhodococcus* sp.	+	+	+
PPT resistant, no utilization of PPT as N source	*Alcaligenes faecalis*	−	+	+
	Alcaligenes denitrificans	−	+	+
	Agrobacterium tumefaciens	−	+	+
	Serratia plymuthica	−	+	+
	Pseudomonas sp.	−	+	+
	Enterobacter sp.	−	−	+
	Enterobacter agglomerans	−	−	+
PPT susceptible	*Escherichia coli*	−	−	+

[a] Cell extracts, showing no PPO formation in the absence of any cofactor, were supposed to be oxidase negative.

DISCUSSION

Three different L-PPT-metabolizing enzymes were found in soil microorganisms: a PPT oxidase, a PPT transaminase, and a PPT *N*-acetyltransferase. The distribution of the enzymes among the investigated strains is summarized in Table 3. Two of them, the oxidase and the transaminase, were also detectable in resting cells of *Rhodococcus* sp.

As products of these enzymes, PPO, the corresponding 2-oxoacid of PPT, and *N*-acetyl-PPT were identified (Fig. 5). The latter compound could not be determined in the resting cell experiments with *Rhodococcus* sp. This does not necessarily mean that N acetylation of PPT is not occurring in living cells. It may be due instead to a very low enzyme activity of the acetyltransferase under the given culture conditions or to a low transportation rate of the acetylated product through the bacterial cell wall. The identification of *N*-acetyl-PPT as one of the major metabolites of Bialaphos (L-phosphinothricyl-L-alanyl-L-alanin) in soil (A. Suzuki, K. Nishide, M. Shimura, T. Watanabe, and J. Yamamoto, Abstr. 6th Intl. Congr. Pesticide Chem., abstr. no. 116, 1986) strongly suggests the presence of a PPT-acetylating capacity in soil microorganisms under natural conditions.

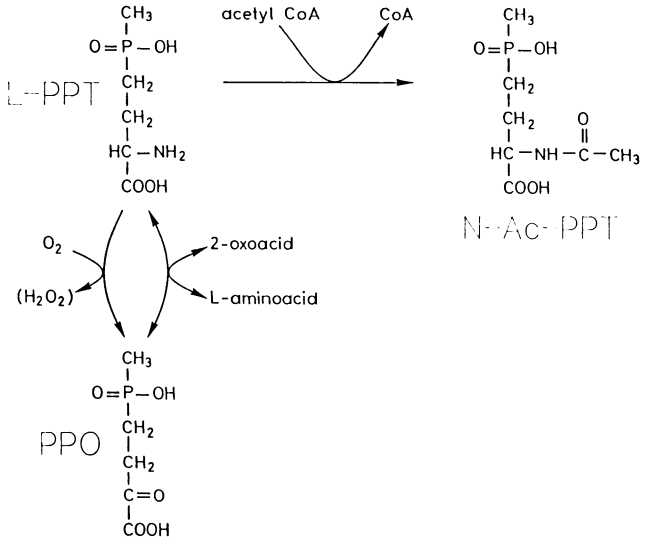

FIG. 5. Proposed initial steps of PPT metabolism by soil microorganisms, derived from data given in this paper.

Earlier investigations showed that, besides PPO, small amounts of the metabolite 2-methyl-phosphinico-propanoic acid, the major degradation product in soil (8), occurred in culture media of *Rhodococcus* sp. at the late stationary phase (19). It is probably formed from PPO by oxidative decarboxylation. The fact that this metabolite was never found as a degradation product of PPT in cell extracts suggests that its formation is due to abiotic rather than enzymatic decarboxylation. On the other hand, PPO-specific decarboxylases might be present in soil microorganisms different from those we selected for in our isolation procedure.

L-Amino acid oxidase enabled *Rhodococcus* sp. to utilize PPT as a source of nitrogen. Cell extracts of cultures grown on PPT were able to deaminate 17 of 20 protein amino acids. Because it is unlikely that a single amino acid induces the synthesis of several amino acid-deaminating enzymes, we assume that the PPT oxidase has a very low substrate specificity. Bacterial L-amino acid oxidases of a low substrate specificity have already been described (4, 6). This property may be crucial for the ability to utilize such a rare amino acid as PPT. This assumption is supported by the fact that the PPT oxidase is inducible by L-glutamic acid as well as by PPT itself (Table 2). Its activity is repressed by NH_4^+ at concentrations above 0.02 mM. Thus, one can expect that in soils with high levels of free NH_4^+, PPT degradation via the oxidase pathway is inhibited.

PPO, the 2-oxoacid analog of PPT, was the degradation product of both the oxidase and the transaminase. The latter enzyme was found to be constitutive. Its presence in cells did not necessarily confer resistance to the herbicide, as shown for *E. coli*. The reason seems to be the ability to perform the synthesis of L-PPT as well. All strains investigated could perform both the formation of PPO from L-PPT and the synthesis of L-PPT from PPO. The reactions are probably catalyzed by the same enzyme. Thus, transamination can proceed in either way, depending on the actual concentrations of substrates (PPT, PPO, amino group donors, 2-oxoacids) within the cell. An enzymatic conversion of PPO to PPT in higher plants, explaining the herbicidal action of the former compound, has already been suggested by Lea et al. (12).

The PPT *N*-acetyltransferase was identified as an enzyme of PPT-resistant soil microorganisms. An enzyme catalyzing a similar reaction is involved in the biosynthesis of the PPT-containing herbicide bialaphos, produced by *Streptomyces hygroscopicus* (10, 14), and it confers resistance to microorganisms and transgenic plants (5, 20). It is not known whether these two enzymes are related. However, in two out of eight PPT-resistant strains (*Enterobacter* sp. and *E. agglomerans*), no PPT *N*-acetylase activity was detectable, indicating that other unknown mechanisms for PPT resistance are likely to exist in microorganisms. Our findings suggest that the initial attack on PPT by soil bacteria is at the amino terminus of the molecule rather than at the C-P bond. This assumption is supported by the fact that so far no microorganisms have been isolated growing on PPT as a sole phosphorus source (19). Thus, the major degradation path-

way for PPT in soil seems to be different from that reported for glyphosate [*N*-(phosphonomethyl)glycine], where bacteria have been isolated, degrading the molecule either from its C or from its P terminus (15, 18).

In field application, PPT concentrations in soil are below 1 mM. Because of the multiple occurrence of PPT-degrading enzymes and the widespread PPT tolerance among microorganisms, we expect that this herbicide will not harm most common soil bacteria. However, with the applied method of isolating bacteria we tested only a small spectrum of the microflora. Further investigations on the response of different soil and rhizosphere microorganisms to PPT and its degradation products would be desirable. Also, the degradation of D-PPT in soil is not yet understood.

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